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Genetically engineered, live, attenuated vaccines protect nonhuman primates against aerosol challenge with a virulent IE strain of Venezuelan equine encephalitis virus☆

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Abstract

Two live, attenuated strains of Venezuelan equine encephalitis virus (VEE), IE1150K and V3526, were administered to macaques to determine if they could elicit protection against an aerosol challenge with virulent VEE virus of the IE variety (VEEV-IE). These viruses were rescued from full-length cDNA clones of 68U201 (VEEV-IE variety) and Trinidad donkey (VEEV-IA/B variety), respectively, and both have a furin cleavage site deletion mutation and a second-site resuscitating mutation. Both vaccines elicited neutralizing antibodies to viruses of the homologous variety but not to viruses of the heterologous variety. Eight weeks after vaccination, the macaques were challenged by aerosol exposure to virulent 68U201. Macaques vaccinated with V3526 were protected as well as macaques inoculated with IE1009, the wild-type infectious clone of 68U201. However, IE1150K failed to significantly protect macaques relative to controls. V3526 has now been shown to protect macaques against both IA/B [Pratt WD, Davis NL, Johnston RE, Smith JF. Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models. Vaccine 2003;21(25–26):3854–62] and IE strains of VEE viruses.
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Keywords: Venezuelan equine encephalitis virus (VEEV); Nonhuman primates; Vaccines

1. Introduction

Venezuelan equine encephalitis viruses (VEEV) are a group of related positive-stranded RNA viruses of the genus *Alphavirus* in the family Togaviridae. By serology, nine varieties have been identified which comprise six subtypes, and these varieties can be further divided into those strains that are associated with epizootics/epidemics (IA/B and IC) and those that are not (enzootic: ID, IE, IF, II, IIIA, IIIB, IIIC, IIID, IV, V, VI) [1]. VEEV is naturally transmitted by mosquitoes with the enzootic strains circulating through rodent and avian hosts in Central and South America [2], and the epizootic strains infrequently evolving from circulating enzootic ID strains [3]. Regardless of the variety, clinical signs of disease caused by VEEV infection in humans are similar and include fever, headache, malaise, and myalgia [4]. Development of encephalitis is more common in the elderly and children, but the disease is rarely fatal (1–5% mortality). Accidents in laboratories first demonstrated that VEEV strains are highly

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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14. ABSTRACT

Two live, attenuated strains of Venezuelan equine encephalitis virus (VEE), IE1150K and V3526, were administered to macaques to determine if they could elicit protection against an aerosol challenge with virulent VEE virus of the IE variety (VEEV-IE). These viruses were rescued from full-length cDNA clones of 68U201 (VEEV-IE variety) and Trinidad donkey (VEEV-IA/B variety), respectively, and both have a furin cleavage site deletion mutation and a second-site resuscitating mutation. Both vaccines elicited neutralizing antibodies to viruses of the homologous variety but not to viruses of the heterologous variety. Eight weeks after vaccination, the macaques were challenged by aerosol exposure to virulent 68U201. Macaques vaccinated with V3526 were protected as well as macaques inoculated with IE1009, the wild-type infectious clone of 68U201. However, IE1150K failed to significantly protect macaques relative to controls. V3526 has now been shown to protect macaques against both IA/B [Pratt WD, Davis NL, Johnston RE, Smith JF. Genetically engineered, live attenuated vaccines for Venezuelan equine encephalitis: testing in animal models. Vaccine 2003;21(25-26):3854-62] and IE strains of VEE viruses.

15. SUBJECT TERMS
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infectious by aerosol which subsequently led to interest in their potential as a biological weapon [4].

Two vaccines developed for protection against the IA/B variety of VEEV are given to at-risk personnel as an investigational new drug (IND). TC-83 is an attenuated strain of VEEV that was obtained after 83 passages in fetal guinea pig heart cells in vitro while C-84 is a formaldehyde-inactivated version of TC-83 used as a boost for waning serum virus-neutralizing (N) antibody titers [5]. Both vaccines have deficiencies [5,6] that necessitated the development of a replacement vaccine, of which V3526 is a lead candidate.

V3526 is a furin cleavage site deletion mutant derived through site-directed mutagenesis of pV3000, an infectious cDNA clone of VEEV-IA/B [7]. The development of V3526 is based on targeting nucleotide sequences thought to be critical for optimal viral replication. In wild-type VEEV, the mature E2 surface glycoprotein is produced by furin cleavage of the PE2 precursor after it forms heterodimers with the E1 glycoprotein. Deletion or mutation of the furin cleavage site in the PE2 glycoprotein results in surface spikes composed of heterodimers of PE2 and E1. Interestingly, the PE2 cleavage signal deletion is lethal to the virus and requires a second site mutation, a Ser for Phe at E1 codon 253 in V3526, for viability. V3526 has been shown to be safe [8] and to protect mice against subcutaneous or aerosol challenge with a virulent VEEV-IA/B [9]. Recently, Pratt et al. [10] reported that out of several vaccine candidates examined, V3526 was best able to protect nonhuman primates against aerosol challenge with a virulent VEEV-IA/B.

Enzootic varieties of VEEV infrequently cause disease in humans and equines [1]. However, epizootic outbreaks of IE viruses among equines have recently occurred in Central America [11], and accidents in laboratories have demonstrated that enzootic VEEV including IE viruses can cause disease in humans [11,12]. Moreover, some laboratory personnel vaccinated with TC-83 or similarly derived, attenuated IA/B vaccines have developed significant signs of disease after aerosol exposure to viruses of the enzootic IE, ID, and IIIA varieties [12–14]. Therefore, a superior new VEEV vaccine would need to protect against aerosol exposure to virulent viruses of both the epizootic or enzootic varieties of VEEV. Because viruses of the IE variety are genetically the most distinct from the IA/B viruses within subtype I, we selected an established aerosol challenge nonhuman primate model using the VEEV-IE strain, 68U201, to test our vaccines [15].

In our study, we tested two strategies. The first strategy examined the ability of V3526 to protect against VEEV-IE. In a earlier study, Hart et al. [16] found that V3526 was sufficient to protect mice against aerosol challenge with 68U201 and that the duration of that protection was greater than 1 year. However, the protection against disease provided by V3526 vaccination did not appear to significantly limit viral replication – these animals developed over a 300-fold increase in serum N antibody titers in response to VEEV-IE challenge. The second strategy examined the ability of IE1150K, a VEEV-IE infectious molecular clone developed in a similar manner to V3526 with a cleavage signal deletion and a second-site resuscitating mutation, to protect against a VEEV-IE challenge. The premise was to evaluate IE1150K in case V3526 was unable to protect nonhuman primates against VEEV-IE and therefore required an IE vaccine component to be used in combination with V3526 for the multivalent VEEV vaccine.

2. Materials and methods

2.1. Animals

Healthy, adult cynomolgus macaques (*Macaca fascicularis*) of both sexes were obtained from the USAMRIID nonhuman primate colony for use in these studies. Before assigning animals to these studies, blood samples from each animal were screened for plaque-reduction neutralization (PRNT) and ELISA antibody for any evidence of previous exposure to VEEV-IA/B, VEEV-IE, VEEV-IIIA, and the western and eastern equine encephalitis viruses. Telemetry implants (Data Sciences International) were implanted subcutaneously on the dorsal surface to monitor temperature and the animals were allowed at least 30 days to recover from surgery, to acclimate, and to produce sufficient telemetry data for baseline modeling of temperature before exposure to VEEV.

2.2. Viruses

Monkeys were given a single subcutaneous (SC) injection of 0.5 ml containing a calculated dose of 10^6 plaque-forming units (PFU) of one of the attenuated viruses, V3526 [7] or IE1150K [17], the infectious IE parent clone IE1009, or virus-free cell culture medium. The challenge virus, 68U201 (VEEV-IE), was isolated in 1968 from the brain of a sentinel hamster in Guatemala [18]. The virus was recovered from a suckling mouse brain suspension and was passaged twice in BHK cells before use. For use in aerosol exposures, the virus was diluted to an appropriate concentration in Hank's buffered saline solution (HBSS) containing 1% fetal bovine serum.

2.3. Aerosol exposures

Before aerosol exposures, macaques were anesthetized by intramuscular injection of Telazol (6 mg/kg) and a whole-body plethysmograph was taken of the animal for 3 min to determine the animal's respiratory capacity. Subsequently, the animal was inserted into a Class III biological safety cabinet located inside a biosafety level-3 suite and exposed in a head-only aerosol chamber to a VEEV aerosol created by a Collison nebulizer for 10 min as previously described [15,19]. Samples were collected from the all-glass impinger (AGI) attached to the aerosol chamber and analyzed by plaque assay to determine the inhaled dose of VEEV.

2.4. Virology and clinical laboratory determinations

Beginning 3 days before exposure and continuing for up to 10 days postexposure, macaques were anesthetized with Telazol (3 mg/kg) and blood samples were collected to assess lymphopenia and viremia. Viremia was measured by plaque assay on Vero cells [19]. Blood cell counts were determined with a Coulter T-series machine and a manual differential count.

2.5. ELISA

ELISA were done using standard ELISA procedures. Briefly, dilutions of primate sera were incubated for 1 h at room temperature on Immulon 2-HB 96-well plates (Thermo Labsystems, Waltham, MA) coated with sucrose-gradient-purified, γ-irradiated VEEV. After washing with PBS-Tween, secondary goat anti-monkey IgG-HRP (Research Diagnostics, Flanders, NJ) was added to the plates and incubated for an additional hour at room temperature, after which the plates were washed again and ABTS substrate (KPL Inc, Gaithersburg, MD) was added for 30 min before plate was read on a Spectramax plate reader (Molecular Devices, Sunnyvale, CA).

2.6. Plaque-reduction neutralization titer

To determine N antibody titers, twofold dilutions of sera starting at 1:20 were mixed with equal volumes of medium containing 200 PFU of virus and incubated overnight at 4 °C. The following day, six-well plates containing confluent monolayers of Vero cells were incubated for 1 h at 37 °C with the virus/antibody mixtures, after which a media/agar overlay was added. Two to three days later, 1 ml of a solution of 5–6% neutral red in 1× HBSS (without phenol red) was added to each well and plaques were counted 24 h later.

2.7. Data analysis

Body temperatures were recorded every 30 min by the DataQuest A.R.T. 2.1 system (Data Sciences). Temperature monitoring began 10–14 days preexposure to develop a baseline training period of temperature data to fit an autoregressive integrated moving average (ARIMA) model [15,19]. Forecasted values for the postexposure time periods were based on the training model extrapolated forward in time. Residual temperature changes postexposure were determined by subtracting the predicted temperature from the actual temperature recorded for each point. Residual temperature changes greater than three standard deviations above the training period were used to compute fever duration (number of hours or days of significant temperature elevation) and fever-hours (sum of the significant temperature elevations).

3. Results

3.1. Response to vaccination

Three groups of eight monkeys were inoculated SC with a single dose of V3526, IE1150K, or IE1009. Back-titration of the inoculum indicated that the doses achieved were 2.5×10^6 for V3526, 5×10^6 for IE1150K, and 2×10^6 for IE1009. A fourth group of eight monkeys was injected with virus-free culture medium as mock-vaccinated controls. A summary of the acute responses to inoculation with the different materials is shown in Table 1. One of the macaques in the IE1150K was removed from the study after vaccination because a review of the animal's medical history revealed that it had been on a prior VEEV study even though no detectable titer was seen during prescreening by ELISA and PRNT. Significant fevers were only seen in monkeys inoculated with IE1009 virus; the post-vaccination fever responses in monkeys receiving V3526 or IE1150K were similar to those seen in the mock-vaccinated monkeys. In addition, only monkeys that received IE1009 had detectable viremia or were positive by throat swab for the presence of virus after vaccination. Lymphopenia was seen in nearly all of the monkeys that were inoculated with a live virus, although the duration and severity was considerably less for monkeys receiving V3526 than those receiving either IE1150K or IE1009.

After vaccination, monkeys were bled once weekly for 7 weeks to monitor the development of antibody to VEEV.

Table 1
Acute response of cynomolgus macaques inoculated with VEEV vaccine candidates

Vaccine group ($n = 8$)	Fever responses[a]				Viral isolation		Lymphopenia[b]		
	T_{max} (°C)	Duration (days)	Duration (h)	Fever-hours (°C h)	Serum	Throat[c]	Number	Duration	% decrease
V3526	2.1	0.1 ± 0.4	17.8	22.6	0/8	0/3	7/8	1.4	−9.2
IE1150K	2.1	0.3 ± 0.8	26.9	33.2	0/7	0/3	6/7	3	−24.2
IE1009	3.1	3.4 ± 2.6	79.3	122.5	8/8	2/3	8/8	4.8	−46.9
Mock	2.5	0.8 ± 1.5	29	39.8	0/8	0/3	3/8	0.8	−8.5

[a] Fever responses: the group means of the maximum temperature elevation (T_{max}); of the number of days (duration [days]) or hours (duration[hours]) monkeys displayed significant temperature elevation; and of the sum of the temperature elevations (fever-hours).

[b] Lymphopenia was evaluated as follows: duration, number of days with a ≥30% drop in blood lymphocyte counts in the 6 days postinoculation compared to baseline values from before inoculation (days −3 to 0); % decrease, the group mean of the average percent decrease in blood lymphocyte counts from baseline during the 6 days postinoculation.

[c] Virus isolation determined by plaque assay; only three animals per group were assessed for virus in the throat.

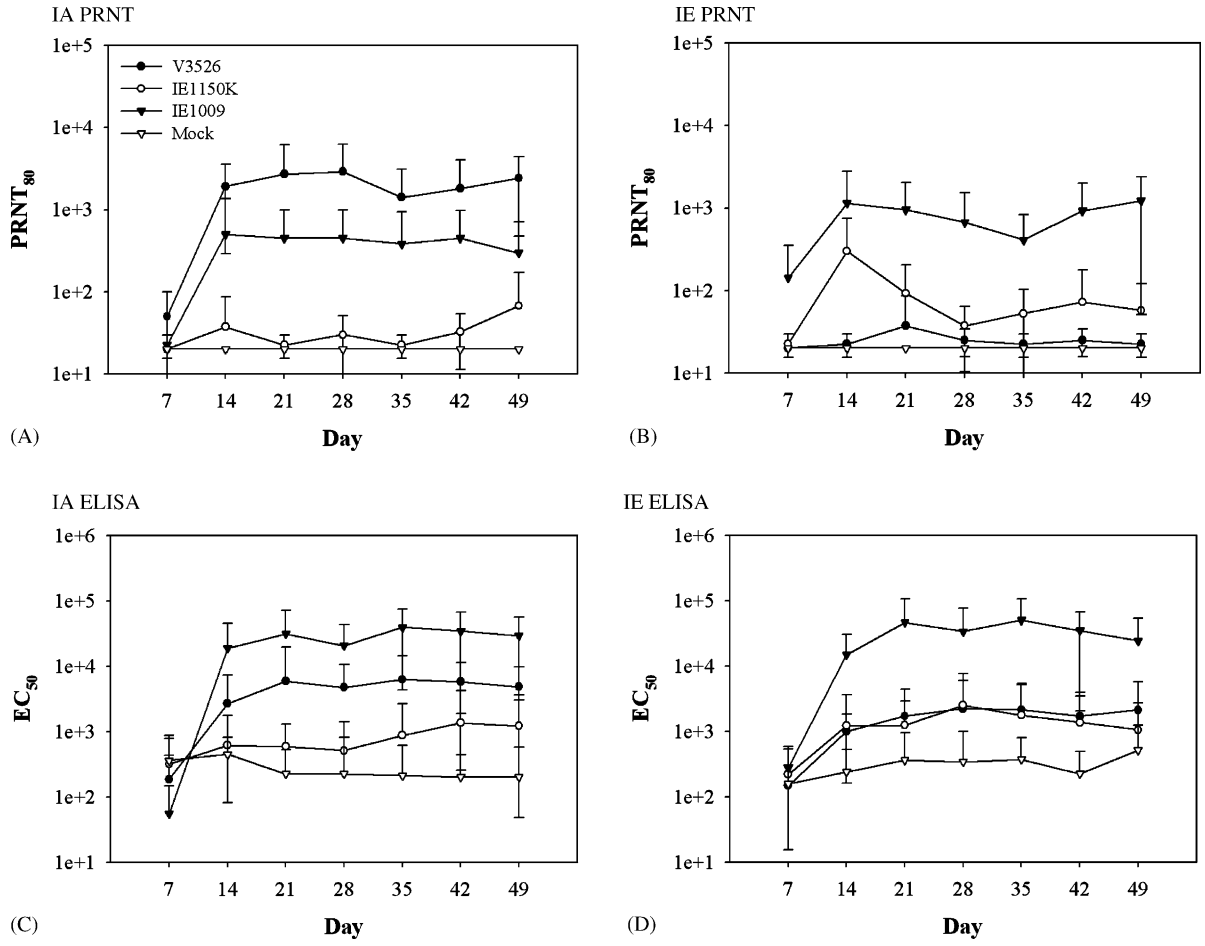

Fig. 1. Increase in antibody titers to IA/B and IE varieties of VEEV after vaccination of macaques with VEEV vaccine candidates. For 7 weeks after vaccination, macaques were bled weekly and their serum was assessed for neutralizing antibody to VEEV by PRNT assay (A, B) and for IgG to VEEV by ELISA (C, D). Antibody responses were measured against IA/B (A, C) and IE (B, D) varieties of VEEV. Neutralizing antibody titers of <40 were calculated as 20. EC_{50} determinations were made by four-parameter logistic modeling of absorbance data in Sigmaplot. Graphs show averaged values for each timepoint with error bars for standard deviation.

Antibody responses could be detected as early as 7 days post-vaccination, with responses peaking between days 14–28 by PRNT and days 21–35 by ELISA (Fig. 1). The peak antibody responses for each group are shown in Table 2. Although all monkeys from the three test groups had antibody responses to both IA/B and IE varieties of VEEV as measured by ELISA, only monkeys inoculated with IE1009 produced significant antibody responses to both varieties as measured by PRNT. V3526 induced an antibody response to IA/B-variety VEEV by both ELISA and PRNT in all eight monkeys, as previously reported [10]. By ELISA, serum IgG titers to VEEV-IE were detectable in all V3526-vaccinated monkeys but were reduced compared to responses to IA/B. Only two of eight monkeys that received V3526 had detectable (≥1:40) N an-

Table 2
Peak antibody titers after vaccination of cynomologus macaques

Vaccine	PRNT[a]		ELISA[b]	
	IA/B	IE	IA/B	IE
V3526	2560 ± 3229 (8/8)	28 ± 49 (2/8)	5120 ± 12627	1487 ± 3677
IE1150K	44 ± 108 (3/7)	87 ± 453 (4/7)	805 ± 2717	861 ± 5055
IE1009	453 ± 858 (8/8)	1280 ± 1575 (8/8)	25520 ± 42924	37077 ± 67072
Mock	20 ± 0 (0/8)	20 ± 0 (0/8)	413 ± 526	458 ± 794

[a] Neutralizing antibody titer values shown are the geometric means of the 80% plaque reduction neutralizing antibody titer. Numbers in parenthesis indicate number of responders with neutralizing titers ≥40 compared to total number of animals in each group. Neutralizing antibody titers of <40 were calculated as 20.

[b] ELISA values reported are the geometric means of the median effective concentration (EC_{50}) as determined by four-parameter logistic analysis of absorbance data.

Table 3
Acute response of cynomolgus macaques to aerosol challenge with VEEV-IE

Vaccine group ($n = 8$)	Fever responses[a]				Viral isolation		Lymphopenia[b]		
	T_{max} (°C)	Duration (days)	Duration (h)	Fever-hours (°C h)	Serum	Throat[c]	Number	Duration	% decrease
V3526	2.1*	0.8 ± 1.5*	32.4*	53.5*	0/8	2/3	6/8	1.9	−14.9
IE1150K	2.7	2.4 ± 2.1	61.6	108.7	0/7	3/3	6/7	2.0	−15.2
IE1009	1.9*	1.3 ± 3.5	34.9*	40.0*	0/8	2/3	2/8	0.2	−7.0
Mock	3.2	3.9 ± 1.2	87.9	149.7	8/8	3/3	8/8	4.6	−47.5

[a] Fever responses: the group means of the maximum temperature elevation (T_{max}); of the number of days (duration [days]) or hours (duration [hours]) monkeys displayed significant temperature elevation; and of the sum of the temperature elevations (fever-hours).
[b] Lymphopenia was evaluated as follows: duration, number of days with a ≥30% drop in blood lymphocyte counts in the 6 days postinoculation compared to baseline values from before inoculation (days −3 to 0); % decrease, the group mean of the average percent decrease in blood lymphocyte counts from baseline during the 6 days postinoculation.
[c] Virus isolation determined by plaque assay; only three animals per group were assessed for virus in the throat.
* Values are significantly different from those seen with mock-vaccinated controls, $p < 0.001$.

tibody to VEEV-IE. Four of eight macaques vaccinated with IE1150K had N titers to the homologous virulent IE virus; however, serum IgG as measured by ELISA to both IE and IA/B varieties of VEEV was detectable in all eight macaques given IE1150K. Overall, antibody responses in IE1150K-vaccinated monkeys were weaker than those seen in either V3526- or IE1009-inoculated monkeys.

3.2. Response to challenge

Eight weeks after vaccination, macaques were challenged by aerosol with 68U201, a virulent strain of VEEV-IE. Previous studies showed that in naive animals exposed by aerosol to VEEV-IE, fever occurred within 36–48 h while viremia and lymphopenia were detectable at 24 h and peaked at 48 h [15]. On the day of challenge, macaques were exposed by aerosol with a dose of $\sim 1 \times 10^8$ PFU of virulent VEEV-IE, which is equivalent to 400 (±200) times the median effective dose required to cause disease. As shown in Fig. 2, mock-vaccinated macaques began to show elevated temperatures as early as 24 h postchallenge, with the fever peaking at day 3 postchallenge and continuing until day 7. Fever duration averaged 87.9 h or 3.9 days with ≥12 h of significant fever for mock-vaccinated macaques (Table 3).

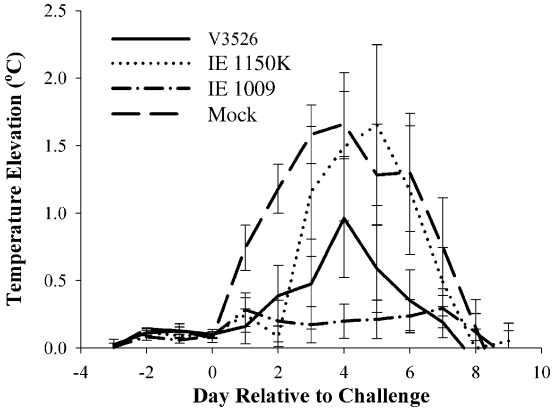

Fig. 2. Fever response in macaques after aerosol challenge with VEEV-IE. Body temperatures were monitored in monkeys aerosol-challenged with VEEV-IE on day 56 after vaccination. Residual temperatures, the difference between the body temperature data, and the forecast values, were averaged over 8 h (2000–0400 h) to eliminate the influence of morning anesthesia on temperature values. The graph shows the mean temperature elevation ± S.D. for V3526-, IE1150K-, IE1009-, or mock-vaccinated macaques beginning on day −3 through day +6 relative to challenge.

Fever onset and duration were different in vaccinated macaques compared to the mock-vaccinated controls. Macaques inoculated with the IE1009 had only a very slight temperature elevation after challenge (Fig. 2), with a fever duration of 34.9 h. Fever onset appears to be delayed 1–2 days in V3526-vaccinated macaques compared to controls, and both the severity and duration were significantly lower. For IE1150K-vaccinated macaques, fever onset was also delayed compared to controls. The duration and severity of the fever after challenge was reduced in these macaques, but not as much as was seen with either IE1009- or V3526-vaccinated macaques and most importantly, not significantly from mock-vaccinated controls.

All of the mock-vaccinated macaques, developed viremia after challenge, with viral titers peaking at $\sim 3 \times 10^5$ PFU on the first day after challenge and persisting for 3 days (Fig. 3 and Table 3). In contrast, no viremia was detected in any of the animals vaccinated with IE1150K, V3526, or IE1009. Throat swabs were taken daily from three macaques in each group and assayed for the presence of virus. Of those so examined, 10 of 12 monkeys had virus present in the throat on the first day after challenge, and each group had at least two monkeys with detectable virus (Table 3). However, there were considerable differences in peak titers and duration of the presence of virus between mock-vaccinated macaques and those macaques vaccinated with VEEV. By day 2 postchallenge, virus was undetectable in the throats of macaques vaccinated with either IE1009 or V3526. Even on day 1, viral titers in the throats of IE1009- or V3526-vaccinated macaques were 4 logs lower than those in controls (Fig. 3). In contrast, viral throat titers in macaques vaccinated with IE1150K were only 1 log lower on day 1 than the titers in mock-vaccinated controls. By day 3, macaques vaccinated with IE1150K were also negative for virus in the throat, whereas virus could be isolated from the throats of mock-vaccinated macaques until day 6.

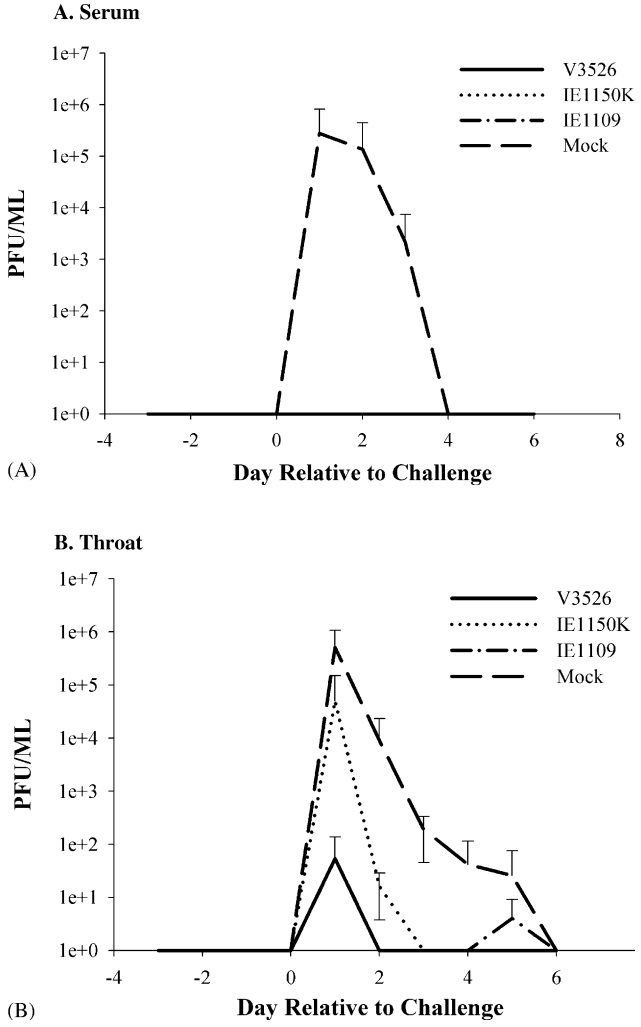

Fig. 3. Virus isolation from the throat and serum after aerosol exposure of macaques to VEEV-IE. Blood samples (A) and throat swabs (B) taken daily from days −3 to +6 were examined for the presence of virus by plaque assay in Vero cells. Graphs show the mean virus concentration in PFU/ml ± S.D. for V3526-, IE1150K-, IE1009-, or mock-vaccinated macaques beginning on day −3 through day +6 relative to challenge.

There were significant differences in lymphocyte counts after challenge between controls and vaccinated macaques. Lymphocyte counts dropped an average of 55% for the mock-vaccinated macaques within 24 h of challenge and these macaques remained lymphopenic throughout the 6 days that the macaques were bled after challenge (Fig. 4 and Table 3). Only two of eight macaques inoculated with IE1009 developed lymphopenia after challenge, and the duration was considerably shorter than that which was seen in the mock controls (0.2 days compared to 4.6 days). Macaques vaccinated with V3526 or IE1150K also had reduced severity and duration of lymphopenia compared to the mock-vaccinated macaques, although not as dramatic as that seen in the IE1009-inoculated macaques.

Twenty-eight days after challenge (day 84 postvaccination), the macaques were bled to assess N antibody titers to

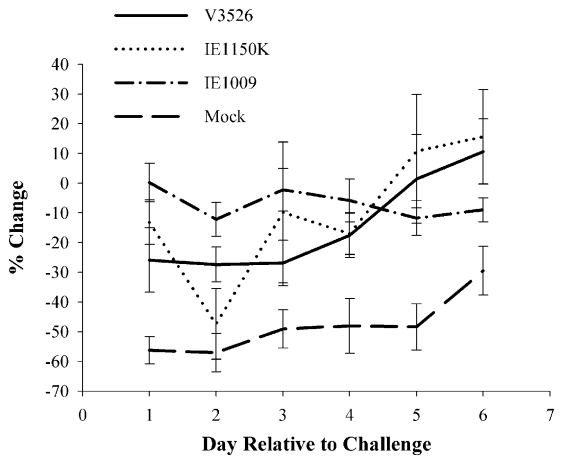

Fig. 4. Lymphopenia after aerosol challenge of macaques with VEEV-IE Macaques were bled daily beginning day −3 to establish baseline lymphocyte counts and assess lymphopenia after challenge with VEEV-IE. The graph shows the mean percent change for each day after challenge relative to prechallenge lymphocyte counts for V3526-, IE1150K-, IE1009-, or mock- vaccinated macaques through day +6 relative to challenge.

VEEV-IE. A comparison of these values to the N antibody titers taken 1 week before challenge (day 49 postvaccination) is shown in Table 4. All groups of macaques exhibited an increase in N antibody titers after challenge, with the increases highest in the mock-vaccinated controls, moderate in the IE1150K- and V3526-vaccinated macaques, and lowest in the IE1009-inoculated macaques.

Peak serum N antibody titers were compared to the signs of disease to determine if these values correlated with protection of the macaques from aerosol challenge with VEEV-IE, as was shown previously for protection against challenge with VEEV-IA/B [10]. We found that serum N antibody titers to VEEV-IE failed to correlate significantly with fever response (Fig. 5A) (Pearson correlation coefficients: duration, $r = -0.43$, $P < 0.0001$; fever-hr, $r = -0.49$, $P < 0.0001$), duration of viremia ($r = -0.47$, $P = 0.0004$), or lymphopenia ($r = -0.45$, $P < 0.0001$). This poor correlation was likely due to the protection observed in V3526-vaccinated macaques that was evident despite the lack of detectable N titers to VEEV-IE before challenge in six of the eight animals vaccinated with V3526. When serum N titers to VEEV-IA/B

Table 4
Fold increase in neutralizing antibody response to VEEV-IE after aerosol challenge of macaques

Day[a]	Vaccine group			
	V3526	IE1150K	IE1009	Mock
49 (−7)	22	40	640	20[b]
84 (+28)	761	780	2792	1174
PRNT increase	35	20	4	59

[a] Day postvaccination (numbers in parenthesis are day relative to challenge).
[b] Neutralizing antibody titers of <40 were calculated as 20.

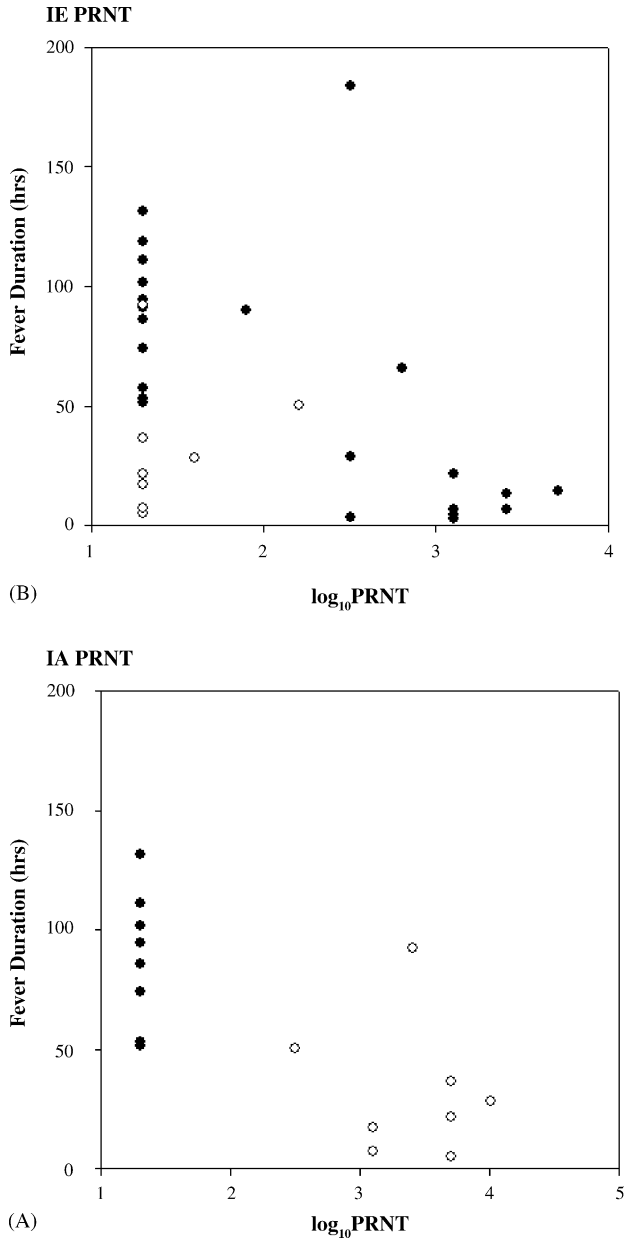

Fig. 5. Correlation between serum neutralizing antibody titers and fever duration after aerosol challenge of macaques with VEEV-IE. Serum neutralizing antibody titers to VEEV-IE (A) correlate with duration of the fever response after VEEV-IE challenge ($n = 31$, $r = -0.43$, $P < 0.0001$). Neutralizing titers to VEEV-IA/B in V3526 and mock-vaccinated macaques (B) also correlated with protection against aerosol challenge with VEEV-IE ($n = 16$, $r = -0.72$, $P < 0.0001$). Clear circles in (A) and (B) are V3526-vaccinated macaques. Closed circles are macaques in either the IE1009-, IE1150K- (A) and mock vaccinated groups (A, B).

from only the mock- and V3526-vaccinated macaques were compared to disease from aerosol challenge with VEEV-IE, a stronger correlation was seen (Fig. 5B) (n = 16, fever duration, $r = -0.72$, $P < 0.0001$; fever-hrs, $r = -0.62$, $P < 0.0001$; duration of viremia, $r = -0.95$, $P < 0.0001$; lymphopenia, $r = -0.71$, $P < 0.0001$).

4. Discussion

The primary objective of this study was to assess the need for a specific VEEV-IE vaccine component to protect against aerosol exposure with the IE strain. If needed, the concept is for this component to be used with V3526, the specific VEEV-IA/B vaccine component, in a multivalent VEEV vaccine format. VEEV-IE was the focus of this study because these strains are the most genetically divergent of the subtype I VEEV varieties and cause disease in humans. Moreover, reports of laboratory infections of individuals vaccinated with TC-83, the live, attenuated VEEV-IA/B vaccine virus currently used under IND, suggests that an additional component would be required for complete protection from VEEV-IE strains. For these reasons, IE1150K was developed as a VEEV-IE component candidate using V3526 as a template. The result was a live, attenuated virus with a furin cleavage site deletion and a second-site resuscitating mutation. Testing in mice showed that IE1150K elicited protection against aerosol and parenteral challenge with a virulent VEEV-IE [17]. We report here a comparison between V3526 and IE1150K, and with the wild-type infectious VEEV-IE clone, IE1009, in their safety and in their ability to protect macaques against aerosol challenge with VEEV-IE. In our comparisons, we used a rigorous and highly refined nonhuman primate model, which showed V3526 and IE1150K to be safe—neither virus caused viremia or significant signs of disease. In contrast, IE1009 caused viremia, significant fever and lymphopenia in the inoculated monkeys.

The results of the aerosol challenge of vaccinated macaques with the wild-type VEEV-IE strain, 68U201, demonstrated that V3526 alone was sufficient to protect against VEEV-IE. Fever duration and severity were essentially identical between the V3526-vaccinated macaques and the IE1009-inoculated macaques. As the infectious clone of 68U201, IE1009 served as a positive control, to provide the best possible protection against subsequent aerosol challenge with a VEEV-IE. Although IE1150K elicited a durable protective immune response in mice [17], it did not elicit protection in macaques to the same level as either IE1009 or V3526. VEEV-specific antibody data (PRNT and ELISA to both IA/B and IE) indicated that the humoral immune responses induced by IE1150K were not as robust as the responses to V3526 or IE1009, supporting other studies in nonhuman primates that showed that the level of serum antibody is key to protecting against VEEV challenge [10,19,20]. Why IE1150K was unable to elicit as vigorous an immune response as V3526 is not clear, as data from mice suggested comparable or better immunogenicity and protection against VEEV-IE regardless of the route of challenge [17]. However, Powers et al. [21] did show using a 68U201 infectious clone that the IE virus is much more sensitive to Type I interferon than is an IA/B virus. This sensitivity tracked with viremia titers and virulence in guinea pigs where the IE virus produced lower levels of viremia and was non-virulent and the IA/B virus produced

higher levels of viremia and was virulent. Based on these results, such differences in interferon sensitivity would be expected between IE1150K and V3526, and could result in lower viral replication and antigen production. This could explain the lower humoral immune response to IE1150K compared to V3526.

The protection conferred against VEEV-IE challenge by V3526 is puzzling given the lack of detectable serum N titers to VEEV-IE before challenge. Because there are detectable N titers to VEEV-IA/B and detectable serum IgG to VEEV-IE in V3526-vaccinated macaques, we see two possible explanations: either the in vitro assay used was unable to measure the in vivo neutralizing response to IE or the antibody response were non-neutralizing (against heterologous VEEV) but cross-protective through some other mechanism. The concept of a non-neutralizing but cross-protective antibody response has been proposed before with alphaviruses [22]; however, the mechanism by which non-neutralizing antibody protects in this instance remains unclear. Further detailed examination of the humoral response in the macaques is needed to better understand how the antibody elicited by V3526 can be cross-protective but non-neutralizing to heterologous VEEV.

We did not examine in this study whether the cellular immune response might be responsible for protection. Prior attempts to measure cytotoxic T-lymphocyte activity in VEEV-vaccinated animals have not been successful [23]. In addition, we saw little indication of a robust $CD8^+$ T cell response in the blood of the macaques postchallenge (data not shown). In one report, T-cell responses contributed to mortality in mice infected with a related alphavirus, Sindbis virus [24]. Antibodies are considered to be the key in both protection and recovery from encephalitis caused by neurotropic viruses including alphaviruses such as VEEV [25]. Further investigation into both the cellular and humoral responses to V3526 is needed to better understand how V3526 was able to mediate protection in the macaques against aerosol challenge with a VEEV-IE.

Because of the ability of VEEV viruses to infect by the aerosol route, there is considerable concern that VEEV could be employed as a biological weapon. Existing vaccines that are given as investigational new drugs (IND) to laboratory personnel may not protect against the range of VEEV viruses that are capable of infecting and causing disease by the aerosol route. It is therefore critical that candidate VEEV vaccines be shown to protect against all varieties and strains that can infect by the aerosol route, particularly those that have broken through the protection afforded by existing IND vaccines. Previously, it was shown that V3526 protects macaques against aerosol challenge with a virulent VEEV of the epizootic IA/B variety [10]. Here, we demonstrate that V3526 also protects macaques against VEEV-IE, the most genetically divergent of the subtype I varieties. These results indicate that a single component, V3526, may be sufficient for protection against the aerosol threat posed by VEEV.

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